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January 6, 2014

Defense Technical Information Center
8725 John J. Kingman Road Ste 0944
Fort Belvoir, VA 22060-6218

Re: ONR Award Number: N00014-09-1-1014

Please find enclosed the Annual/Final Technical Report SF298 for the following award. Thank you for funding Dr. Zuo's and St. Jude's research.

PI: Jian Zuo, Ph.D
Organization: St. Jude Children's Research Hospital
ONR Award Number: N00014-09-1-1014
Award Title: *Hearing Restoration in Mouse Models with Noise-induced Hearing Loss*

If you need any additional information, please contact me at (901)-595-4088.

Sincerely,

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Final Technical Report (12.19.2013)
PI: Jian Zuo, Ph.D.
Organization: St. Jude Children's Research Hospital
ONR Award Number: N00014-09-1-1014
Award Title: Hearing Restoration in Mouse Models with Noise-induced Hearing Loss

A. Scientific and Technical Objectives

As a long-term goal, we aim to develop therapeutics which would restore hearing in navy servicemen who are suffering from noise induced hearing loss (NIHL). Our **central hypothesis** is that inactivation of $p16^{Ink4a}$ or other cell cycle inhibitors in mammalian supporting cells (SCs) will allow them to respond to acoustically damaged hair cells (HCs) and to reenter the cell cycle; subsequently the introduction of Atoh1 will cause the newly created SCs to transdifferentiate into HCs. To test this hypothesis, we propose to develop mouse models and small molecule inhibitors to: **Aim 1**) characterize the regenerative capacity of SCs in mice after noise-induced damage and transient or permanent inactivation of $p16^{Ink4a}$; **Aim 2**) assess the ability of Atoh1 to transdifferentiate SCs into HCs after noise-induced damage in mice. Our studies will provide a basis for future clinical trials using cell cycle inhibitory and HC-differentiation promoting drugs to restore hearing in humans. Since the award, we have modified **Aim 1** to include screening of $p27^{Kip1}$ inhibitors; this is based on our recent exciting results of acute inactivation of $p27^{Kip1}$ in neonatal SCs.

B. Approach

We plan to develop mouse genetic models and small molecule inhibitors to achieve the following proposed aims:

Aim 1A: To determine the regenerative capacity of SCs in $p16^{Ink4a}$-null mice after noise-induced damage.

Aim 1B: To transiently inactivate $p16^{Ink4a}$ after noise-induced damage.

Aim 1C: To develop small molecule inhibitors of $p16^{Ink4a}$ and $p27^{Kip1}$.

Aim 2A: To create and characterize transgenic mice with inducible overexpression of Atoh1 in postnatal and adult SCs and measure the effects of Atoh1 overexpression after noise-induce damage.

Aim 2B: To test the ability of γ-secretase inhibitors to increase Atoh1 expression in the mouse cochlea and measure their effects on HC morphology and hearing before and after noise-induced HC damage.

C. Concise Accomplishments

We have discovered in vivo that:

1. Noise-induced HC damage causes an increase of proliferating cells in the organ of Corti of adult $p16^{Ink4a}$ knockout mice; the cell origin of these proliferating cells remains to be determined.

2. For the first time and in contrast to common belief, postnatal mammalian cochleae have the capacity to regenerate auditory HCs after damage, similar to non-mammalian auditory sensory epithelia; such capacity declines with age.
3. Sox2 directly regulates $p27^{Kip1}$ to maintain quiescence of postmitotic cochlear SCs; such roles decline with age.
4. SCs in the mammalian cochlea can be converted to sensory HCs in mouse models with a single factor Atoh1; such ability declines with age.
5. Activation of Notch1 signaling can induce ectopic sensory HCs in an age–dependent manner.
6. $p27^{Kip1}$ inactivation in neonatal HCs results in proliferation and survival of HCs and preservation of functional hearing in mice.
7. Synergistic roles of Atoh1 and β-catenin in neonatal Lgr5+ supporting cells in mice.
8. Combination of Atoh1 activation and $p27^{Kip1}$ inactivation in supporting cells led to new HC formation in adult mouse cochleae.
9. Combination of Atoh1 and β-catenin activation in supporting cells yielded new HCs in adult mouse cochleae
10. Chemical compounds identified in our primary screen have potential to inhibit $p27^{Kip1}$, a key molecule in controlling regeneration of HCs.

Our exciting findings will allow us to further decipher the mechanisms and to develop small molecule inhibitors for restoration of hearing in Navy servicemen suffering from NIHL.

D. Expanded Accomplishments

Aim 1A: To determine the regenerative capacity of SCs in $p16^{Ink4a}$-null mice after noise-induced damage.

Similar to other tissues, we found that $p16^{Ink4a}$ is expressed at low levels in the cochlea of young animals and expression increases with age. We also found that $p16^{Ink4a}$-null mice have normal hearing and HC morphology at 1, 2 and 3 months of age. We have successfully damaged OHCs, but preserved IHCs, with noise exposure in adult mice on either mixed or C57Bl/6 strain background. For the first time, we have detected a significant increase in BrdU+ cells within the organ of Corti of the $p16^{Ink4a}$-null mice on the mix background compared to wild-type controls. Interestingly, most BrdU+ cells are close to IHCs and thus could be inner PCs or IPHs. Also these BrdU+ cells are not immune cells because they do not co-label with CD45. We are performing definitive lineage tracing experiments to confirm that these BrdU+ cells are indeed proliferating SCs. These results provide the first evidence that $p16^{Ink4a}$ plays a critical role in mammalian HC regeneration after NIHL (Cox et al., in preparation).

Non-mammalian vertebrates such as birds, fish and amphibians can regenerate hair cells (HCs) after damage. In contrast, damage to auditory HCs, caused by noise exposure or other factors, is currently believed to be permanent in humans and other mammals. However, the absence of HC regeneration in mammals has only been confirmed in adults.

We have recently developed a novel method to damage HCs in the neonatal, mouse cochlea *in vivo* and observed spontaneous HC regeneration in the less mature, apical turn of the cochlea. Regenerated HCs are similar to endogenous HCs expressing four HC markers, including prestin, a terminal differentiation marker specific to outer HCs. By lineage tracing, we confirmed the mechanisms of HC regeneration where SCs proliferate and transdifferentiate into HCs. We also defined a critical period when cochlear HC regeneration can occur (Cox et al., *Development, in press*).

Aim 1B: To transiently inactivate $p16^{Ink4a}$ after noise-induced damage.

We have successfully created p27-IRES-rtTA knockin mice. When crossed with two reporter lines (Tet-on-LacZ and Tet-on-Cre; Rosa26-YFP), we did not observe any reporter activity in the cochlea or cerebellum after induction with various doses of Doxycyclin in late embryonic and postnatal mice. Even after removing the neo marker from the p27-IRES-rtTA locus and breeding homozygotes of p27-IRES-rtTA to enhance the level of rtTA, we failed to observe reporter expression in SCs. Given that the reporter worked as expected under another rtTA line, we believe that the rtTA level is not high enough because of p27 level is generally low in postnatal SCs. Instead, we have characterized Sox10-rtTA which labels SCs at neonatal ages. We have thus developed an alternative rtTA mouse line that will be extremely useful for HC regeneration studies. We believe that the phenotypes in $p16^{Ink4a}$-null mice after NIHL remain further characterized, and we are therefore putting on hold the studies of transient inactivation of $p16^{Ink4a}$.

Aim 1C: To develop small molecule inhibitors of $p27^{Kip1}$.

We have successfully deleted each of three key molecules (Sox2, $p27^{Kip1}$ and retinoblastoma protein) in neonatal SCs within the organ of Corti and observed subsequent SC division; unfortunately, we did not find new sensory HCs in either model (Yu et al., J. Neurosci. 2010; Liu and Walters, et al., J. Neurosci., 2012). However, when we deleted $p27^{Kip1}$ in neonatal HCs, we surprisingly observed proliferation and survival of HCs and preservation of hearing in adult mice (Walters et al., in preparation).

Given the importance of $p27^{Kip1}$ and its regulation in HC regeneration, we are employing fragment based screening techniques to screen for inhibitors of $p27^{Kip1}$ that will allow cyclin dependent kinases (CDKs) to perform their catalytic activity within the cell division. From the preliminary NMR (WaterLOGSY) screening and 2D ^{1}H-^{15}N HSQC verification, we have identified two regions within p27KID that bind preferably to two sets of fragment compounds. One of these regions is responsible for inhibition of the ATP catalytic pocket of the CDKs. Using cheminformatics, we have identified another set of new compounds in the St. Jude >0.5 million compound library that have higher molecular weight and similar core structure that exhibits improved affinity for p27KID. These studies provide promising preliminary results for developing inhibitors of p27 for HC regeneration (Iconaru et al., in preparation).

In parallel, we undertook an unbiased large scale screen of >4,385 unique bioactive compounds for transcriptional inhibitors of $p27^{Kip1}$. This approach has been fruitful in that four top compounds have been identified and characterized in HeLa, MEF, HEK cells with potent inhibitory effects and minimal toxicity. Moreover, we showed that our top compound (A2CE) exhibits inhibition on p27 transcript in neonatal mouse cochlear explant culture. In addition, we identified a molecular target that mediates such inhibition of A2CE (Walters et al., submitted). We are currently testing these compounds in ex vivo and in vivo in mouse models.

Aim 2A: To create and characterize transgenic mice with inducible overexpression of Atoh1 in postnatal and adult SCs and measure the effects of Atoh1 overexpression on SCs after noise-induce damage.

When we acutely expressed Atoh1-HA in postnatal SCs using the newly generated transgenic mouse and various CreER mouse lines, we observed SC-derived new sensory HCs in the postnatal mouse cochlea. Pillar and Deiters' derived new HCs are immature (Liu et al., J. Neurosci., 2012). Interestingly, new HCs derived from inner phallengeal (IPh) cells appear mature IHCs with multiple HC markers, IHC morphology and nerve innervations, although vGlut3 or prestin is not expressed. Moreover, we analyzed the electrophysiological properties of these IPh-derived new IHCs at various postnatal ages, they exhibit expected K-currents albeit smaller (Liu et al., submitted). These results provide strong evidence that Atoh1-mediated therapeutic approach may still be useful in regenerating new IHCs to restore some hearing, although these new IHCs are not fully mature and similar to PC and DC-derived new HCs, other factors are needed in addition to Atoh1.

To overcome the major hurdle in HC regeneration of adults, we tested combinatory genetic manipulations in adult cochlear SCs because individual manipulations did not yield any responses. To our surprise, when Atoh1 activation and p27Kip1 inactivation were combined in adult SCs, we observed new HCs in two independent mouse models of CreER (Plp-CreER and Fgfr3-CreER). Moreover, when Atoh1 and β-catenin were both activated in adult SCs, we also observed new HCs. These new HCs are also in ~150 per cochlea albeit immature. In addition, we are exploring various mechanisms for these exciting observations. Our breakthrough efforts are in parallel to in vitro HC generation from iPS/ES cells and to regeneration of heart cardiomyocytes or pancreas β-islet cells in that newly regenerated cells are also immature and in small numbers.

Aim 2B: To test the ability of γ-secretase inhibitors to increase Atoh1 expression in the mouse cochlea and measure their effects on HC morphology and hearing before and after noise-induced HC damage.

We have shown that reactivation of Notch signaling in the developing mouse cochlea resulted in ectopic HCs. However, such ability declines with age (Liu et al., PLoS One, 2012; Liu et al., Dev Dyn., 2012). These results demonstrate that Notch activation alone is insufficient to induce new HC regeneration in adult cochleae. However, given our Atoh1 overactivation results, we are testing if the combination of Atoh1 and Notch activation would result in HC regeneration with larger numbers and more maturity. Furthermore, we have shown that Wnt signaling plays a key role in SC proliferation but not transdifferentiation (Chai and Kuo wet al., PNAS 2012). Therefore, the combination of Wnt and Atoh1 is another promising avenue to regenerate HCs in postnatal and adult cochleae (Kuo et al., in preparation). We will test the drugs proposed here (γ-secretase inhibitors) after these in vivo genetic studies. Similar studies have been performed in another lab and results were published despite caveats (Mizutari et al., Neuron 2013). The principal one is that new HCs if any cannot be definitively shown to be derived from SCs because Sox2-CreER labels HCs when induced at P0-2 (Zuo, unpublished). These results are therefore questionable and warranted to be further replicated. We are in a unique position to replicate and further our understanding of the involvement of Notch, Atoh1 and Wnt in HC regeneration in adult cochleae.

E. Major Problems/Issues and Lessons Learned

In general, our proposed aims have been largely achieved and additional goals have been achieved as well. As results, we have published 24 publications on this subject and clearly played a leading role in this research area. These studies had also allowed us to expand our program into several additional areas that have been further funded by ONR, DURIP and potentially Army. We are grateful for ONR's generous support.

Our efforts to regenerate HCs in mouse cochleae have yielded significant results in the field. However, significant obstacles remain. First, conversion rate from SCs to HCs is limited (<20% with <150 new HCs per cochlea); second, new HCs are immature thus not functional. This is not surprising because our breakthrough efforts are in parallel to in vitro HC generation from iPS/ES cells and to regeneration of heart cardiomyocytes or pancreas β-islet cells in that newly regenerated cells are also immature and in small numbers. We are exploring new approaches to identifying factors or drugs that promote HC maturation.

Another major obstacle for HC regeneration in mammalian cochleae is that mature cochleae failed to respond to various individual genetic manipulations (i.e., p27 inactivation, Atoh1 or β-cat ectopic expression), but when combined, responded well. This resembles the four transcription factors discovered for iPS cells and suggests that further combination of more than two may work better for HC regeneration and new combinations need to be identified for efficient HC regeneration.

We have successfully developed two independent drug screens for p27 inhibitors: fragment-based screens and luciferase reporter based screens. Among the top compounds, the top transcriptional inhibitor compound has been tested for cochlear effects, highlighting its potential in preclinical testing (mouse models in vivo) in the future. Drug screens and mouse models are both important to drug discovery and development, an area we will focus on the future.

Several projects have not been fruitful during the course of this grant. $p16^{Ink4a}$ had not given us significant results as planned; this is most likely due to the compensation of other genes. γ-secretase inhibitors had not been successful in our hands, despite another report; their results remain to be further validated in our new models.

F. Technology Transfer

- We have contacted Sound Pharmaceuticals, Inc (Dr. J. Kil, another ONR awardee) on issues related to HC regeneration and ototoxicity in the past two years and will keep in touch on possible transfer of our mouse strains for their research. We also would like to share our results on p27 inhibitor screens with them for future development of drugs.

- We have been contacted by CFD Research Corporation (Dr. Andrzej Przekwas, another ONR awardee) who is interested in visiting us for future collaboration. I have served as scientific consultant for their new application to ONR.

- We have collaborated with Fate Therapeutics Inc. (Dr. Scott Thiers) on Atoh1 activators and $p27^{Kip1}$ inhibitors for HC regeneration.

- We have filed a provisional patent application through St. Jude Children's Research Hospital on the combinatory use of Atoh1 activators and $p27^{Kip1}$ inhibitors for HC regeneration in adults.

G. Foreign Collaborations and Supported Foreign Nationals

We have hired several foreign nationals in our group as St. Jude employees using this ONR grant to work on this project.

- Dr. Luigi Iconaru, an experienced postdoc fellow with chemistry background and a Romanian national, is screening for small molecule inhibitors using NMR. He has made significant progress since he joined the lab in April 2010 and had since been awarded a prestigious postdoc fellowship award by St. Jude.
- Mr. Zhiyong Liu, an excellent PhD graduate student and a Chinese national, has been working on mouse models of Atoh1, p27 and Notch. He

has made significant discoveries on all these areas and is a major contributor to the ONR funded project. He has graduated in Aug. and further stayed on continuing on his ongoing studies of HC regeneration until April 25, 2012.

- Ms. Lingli Zhang, a technician and a Chinese national, was hired in Aug. 2009 and has been working closely together with Dr. Cox on $p16^{Ink4a}$ and DTA and helping others for genotyping. She is an important member on all ONR funded projects.

No collaboration with foreign institutes/individuals.

H. Publications supported in part by this grant award:

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